Fostriecin, an Inhibitor of Protein Phosphatase 2A, Limits Myocardial Infarct Size Even When Administered After Onset of Ischemia

Christof Weinbrenner, MD; Christopher P. Baines, BS; Guang-Shung Liu, MD; Stephen C. Armstrong, PhD; Charles E. Ganote, MD; Aimée H. Walsh, BS; Richard E. Honkanen, PhD; Michael V. Cohen, MD; James M. Downey, PhD

**Background**—The role of protein phosphatases (PPs) during ischemic preconditioning in the rabbit heart was examined.

**Methods and Results**—Fostriecin, a potent inhibitor of PP2A, was administered to isolated rabbit hearts starting either 15 minutes before or 10 minutes after the onset of a 30-minute period of regional ischemia and continuing until the onset of reperfusion. After 2 hours of reperfusion, infarct size was measured with triphenyltetrazolium chloride. In a second study with isolated rabbit cardiomyocytes, the effect of fostriecin pretreatment was assessed by measuring changes in cell osmotic fragility during simulated ischemia. PP1 and PP2A activities of isolated control and ischemically preconditioned cells were also measured. In a third series of experiments, left ventricular biopsies of isolated rabbit hearts were obtained before and at selected times during 60 minutes of global ischemia, and the tissue was assayed for PP1 and PP2A activities. In isolated hearts pretreated with fostriecin, only 8% of the ischemic zone infarcted, significantly less than that in untreated control hearts (33%; \( P < 0.001 \)) but comparable to that in ischemically preconditioned hearts (9%; \( P < 0.001 \) versus control). Significant protection was also observed in the hearts treated only after the onset of ischemia (18% infarction; \( P < 0.05 \) versus control). In isolated myocytes, fostriecin also provided protection comparable to that produced by metabolic preconditioning. Preconditioning had no apparent effect on the activity of either PP1 or PP2A in isolated ventricular myocytes or ventricular tissue obtained from heart biopsies.

**Conclusions**—Fostriecin, a potent inhibitor of PP2A, can protect the rabbit heart from infarction even when administered after the onset of ischemia. But inhibition of either PP1 or PP2A does not appear to be the mechanism of protection from ischemic preconditioning. (*Circulation.* 1998;98:899-905.)

**Key Words:** ischemia, phosphorylation, protein phosphatases, fostriecin
cytes to determine whether either enzyme is inhibited in ischemically preconditioned hearts.

Methods
All procedures were approved by the Institutional Animal Care and Use Committee and were in conformance with recommendations published in the Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, 1996.

Perfused Rabbit Heart
New Zealand White rabbits of either sex were anesthetized with intravenous sodium pentobarbital (30 mg/kg) and ventilated with 100% oxygen. The heart was excised and quickly mounted on a Langendorff apparatus (transfer time, ≤60 seconds). The heart was perfused at constant pressure (100 cm H2O) with a Krebs-Henseleit buffer containing (in mmol/L) NaCl 118.5, KCl 4.7, MgSO4 1.2, CaCl2 2.5, NaHCO3 24.8, KH2PO4 1.2, and glucose 10. The perfusate was gassed with 95% O2/5% CO2, and the perfusate temperature was maintained at 38°C. A saline-filled latex balloon was inserted into the left ventricle. Balloon volume was adjusted to provide a left ventricular end-diastolic pressure of 5 to 10 mm Hg.

Measurement of Infarct and Risk Zones
A suture was placed around a prominent branch of the left coronary artery, and the ends were pulled through a small vinyl tube to form a snare. The coronary branch was occluded by tightening the snare. Reperfusion was confirmed by enhanced coronary flow. Infarction was induced by 30 minutes of regional ischemia, which was then followed by 2 hours of reperfusion.

At the end of the experiment, the coronary artery was reoccluded, and 1- to 10-μm zinc cadmium sulfide fluorescent particles (Duke Scientific Corp) were infused into the perfusate to demarcate the risk zone as the tissue without fluorescence. The heart was then cut into slices 2 mm thick. The slices were incubated in 1% triphenyltetrazolium chloride in pH 7.4 phosphate buffer for 20 minutes at 37°C. The areas of infarct (triphenyltetrazolium chloride-negative tissue) and risk zone (nonfluorescent under ultraviolet light) were determined by planimetry. Infarct and risk zone sizes were then calculated by multiplying each area by the slice thickness and summing the products. Infarct size was expressed as a percentage of the risk zone.

Experimental Protocols: Infarct Size Studies
Five groups with 6 hearts in each group were studied. Group 1 served as controls and received only 60 minutes of global ischemia followed by 10 minutes of reperfusion before the onset of 30 minutes of regional ischemia. In the third group, 3.4 μmol/L ascorbate was infused over a period of 45 minutes, starting 15 minutes before the onset of ischemia and continuing through the ischemic period. This group was included because the stock solution of fostriecin contained ascorbate as an antioxidant. The concentration of ascorbate infused in group 3 was the same as that present in a solution containing 1 μmol/L of fostriecin. In group 4 (Fos-Pre), 1 μmol/L of fostriecin (NSC 339638, lot 700-95-202, kindly provided by the Division of Cancer Treatment, National Cancer Institute and Parke-Davis Pharmaceuticals) was infused according to the same protocol as that for ascorbate in group 3. In the fifth group (Fos-Post), 10 μmol/L fostriecin was infused over a period of 20 minutes, beginning 10 minutes before the onset of ischemia and continuing until reperfusion.

Cardiomyocyte Isolation
As previously detailed, rabbit ventricular myocytes were isolated by including collagenase ( Worthington Biochemical Corp) in a calcium-free perfusate for 15 minutes and then macerating the heart. Viable myocytes were separated by slow-speed centrifugation in buffer containing 1% BSA. Cells were made calcium-tolerant by slowly restoring the calcium in the medium to 1.25 mmol/L. Four hearts were used to isolate fresh myocytes for the osmotic fragility studies. Cells from each isolate were divided into 6 tubes for the osmotic fragility study. Each tube received a different treatment, and all 6 tubes were studied in parallel. Ischemia was simulated by centrifuging myocytes into a pellet (≈0.5 mL of packed cells), and the supernatant was replaced with 0.5 mL of mineral oil to exclude oxygen. Every 30 minutes for 3 hours, a 25-μL aliquot of cells was obtained with a pipette for determination of viability/fragility by observing whether the cells could exclude trypan blue dye when diluted in a hypotonic (85 mM) medium. Cells unable to exclude the dye were considered to have experienced membrane failure from the osmotic stress. During simulated ischemia, there is a progressive increase in osmotic fragility that occurs at a very predictable rate, and preconditioning delays that process. A plot of % stained cells versus time was constructed, and an index of fragility was calculated as the area under the curve and presented as % · h.

Experimental Protocols: Cardiomyocyte Studies
Cardiomyocytes were preconditioned by incubation in glucose-free medium for 10 minutes, after which glucose was restored for 30 minutes. Subsequently, the cells were pelleted for 180 minutes. Control cells received only the prolonged period of simulated ischemia. Either fostriecin (10 μmol/L) or okadaic acid (10 μmol/L) was added to myocytes 15 minutes before simulated ischemia. Control and preconditioned myocytes were also incubated in oxygenated buffer for 210 minutes and were not pelleted. All 6 conditions were tested simultaneously in each of 4 replications. In addition, for reference purposes, the effects of ascorbate on ischemic myocytes and of fostriecin on oxygenated cells were examined in separate cell isolates.

PP measurements were performed in cardiomyocytes from 5 different isolations. Both untreated and preconditioned cells were evaluated. For these biochemical studies, cardiomyocytes were ischemically preconditioned by pelleting the cells for 10 minutes and then resuspending them in oxygenated buffer for 15 minutes. Control cells received only the prolonged period of simulated ischemia. Samples of myocytes were obtained before and after 15, 30, 60, and 90 minutes of simulated ischemia. In oxygenated control and preconditioned myocytes that were not pelleted for 90 minutes, samples were taken only at times 0 and 90 minutes. In an additional experiment, we measured PP activity in cells treated with fostriecin. Cells were incubated in 1 or 10 μmol/L fostriecin for 15 minutes before the cells were processed. Control cells were incubated in 3.4 and 34 μmol/L ascorbate (the vehicle for fostriecin) for 15 minutes. The samples were sonicated and then centrifuged for 10 minutes at 15 000g. The supernatant was used for PP measurements.

Experimental Protocols: PP Measurements in Left Ventricular Biopsies
For the biochemical studies, we used a globally ischemic heart, which allowed us to obtain multiple biopsies from each heart. The control hearts (n = 6) received only 60 minutes of global ischemia, whereas the preconditioned hearts (n = 6) were subjected to 5 minutes of global ischemia followed by 10 minutes of reperfusion before the onset of the 60-minute period of ischemia. Five transmural biopsies (35 to 99 mg each) were taken from the left ventricular free wall of each heart with a motor-driven biopsy tool and immediately frozen in liquid nitrogen. The first biopsy was taken after the 20-minute equilibration period and the second just before the onset of the 60-minute period of global ischemia. Subsequent biopsies were taken after 10, 30, and 60 minutes of global ischemia.

Tissue Preparation
Biopsies were weighed and homogenized with a beadbeater (Mini-Beadbeater, Biospec Products) 3 times for 10 seconds each in 600 μL of buffer chilled to 4°C containing Tris-HCl 50 mmol/L (pH 7.4) and EDTA 1 mmol/L. Subsequently, the samples were centrifuged at 1000g for 5 minutes. The pellet was discarded, and the protein content of the supernatant was determined according to the method of Bradford.
TABLE 1. Hemodynamic Data for Infarct Size Study

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>Treatment</th>
<th>Occlusion</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR, bpm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>216±6</td>
<td>214±8</td>
<td>210±7</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>6</td>
<td>213±8</td>
<td>209±6</td>
<td>211±9</td>
<td>210±9</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>6</td>
<td>211±2</td>
<td>201±1*</td>
<td>200±0*</td>
<td>200±0*</td>
</tr>
<tr>
<td>Fos-Pre</td>
<td>6</td>
<td>203±2</td>
<td>203±2</td>
<td>200±0</td>
<td>200±0</td>
</tr>
<tr>
<td>Fos-Post</td>
<td>6</td>
<td>214±8</td>
<td>204±2</td>
<td>200±0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DP, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>137±6</td>
<td>75±7*</td>
<td>109±9*</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>6</td>
<td>116±3†</td>
<td>96±7*</td>
<td>50±7*</td>
<td>74±4*</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>6</td>
<td>120±4</td>
<td>107±6</td>
<td>40±6*</td>
<td>64±4*</td>
</tr>
<tr>
<td>Fos-Pre</td>
<td>6</td>
<td>126±2</td>
<td>122±3</td>
<td>71±4*</td>
<td>90±3*</td>
</tr>
<tr>
<td>Fos-Post</td>
<td>6</td>
<td>112±5†</td>
<td></td>
<td>63±6*</td>
<td>93±5*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CF, ml/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>59±3</td>
<td>32±8*</td>
<td>44±5*</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>6</td>
<td>68±5</td>
<td>71±6</td>
<td>39±5*</td>
<td>46±4*</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>6</td>
<td>55±3</td>
<td>44±3*</td>
<td>21±4*</td>
<td>32±3*</td>
</tr>
<tr>
<td>Fos-Pre</td>
<td>6</td>
<td>57±3</td>
<td>49±2*</td>
<td>28±2*</td>
<td>40±2*</td>
</tr>
<tr>
<td>Fos-Post</td>
<td>6</td>
<td>52±3</td>
<td>30±4*</td>
<td>40±2*</td>
<td></td>
</tr>
</tbody>
</table>

HR indicates heart rate; PC, ischemic preconditioning; Fos-Pre, fostriecin (1 μmol/L) infused for 45 minutes, starting 15 minutes before the 30-minute occlusion; Fos-Post, fostriecin (10 μmol/L) infused for 20 minutes, starting 10 minutes into the 30-minute occlusion; DP, left ventricular developed pressure; CF, coronary flow, and n, number of animals in each group.

*P<0.05 vs baseline; †P<0.05 vs control.

Preparation of Phosphoprotein Substrate

[32P]-labeled histone with a specific activity >4.5×10^6 dpm/nmol of incorporated phosphate was prepared by phosphorylation of bovine brain histone (type 2AS from Sigma) with cAMP-dependent protein kinase (from rabbit muscle) in the presence of [γ-32P]ATP as previously described.12

Determination of PP Activity

Tissue and cell PP activities were determined in duplicate by quantification of liberated 32P from [32P]histone after incubation with the samples.12 PP1 and PP2A were assayed in aliquots of homogenate and sonicated cell supernatant in the absence of divalent cations in 80 μL of buffer (final volume) containing (in mmol/L) Tris 50 (pH 7.4), DTT 5, and EDTA 1. The reaction was initiated by addition of 2 μmol/L of [32P]histone (based on incorporated 32P) and was conducted at 30°C for 10 minutes. The reaction was stopped by the addition of 100 μL of 1N H2SO4 containing 1 mmol/L K2HPO4, and 32P, liberated by the enzymes was extracted as a phosphomolydbate complex.9,12 Briefly, free phosphate was extracted by adding 20 μL of ammonium molybdate (7.5% wt/vol in 1.5N H2SO4) and 250 μL of isobutanol:benzene (1:1, vol:vol) to each tube. The tubes were mixed vigorously for ~10 seconds followed by centrifugation at 14 000g for 2 minutes. Radioactivity of a 100-μL aliquot from the upper phase was quantified with a scintillation counter. Discrimination between PP1 and PP2A was performed by adding 2 nmol/L okadaic acid to the assay mixture. Okadaic acid inhibits both PP1 and PP2A activity in a concentration-dependent manner (IC50 for PP1, 15 to 50 nmol/L; IC50 for PP2A, ~0.1 nmol/L).13,14 Residual PP activity in the presence of 2 nmol/L okadaic acid was considered to be PP1-like activity, and PP2A-like activity was calculated by subtraction of PP1-like activity from the total activity determined in the absence of okadaic acid. The assay buffer contained 1 mmol/L EDTA, which chelates divalent cations, thus inhibiting PP2B and PP2C.

Statistics

Values are presented as mean±SEM. One-way ANOVA with repeated measures and Tukey-Kramer post hoc tests were used to test for differences within groups and, without repeated measures, to test for differences in infarct size and areas under curves (Instat, Graphpad Software). A multivariate repeated-measures analysis was performed to test for time-related and group-related differences in the myocardial biopsy data (Systat). A value of P<0.05 was considered significant.

Results

Infarct Size Studies

With the exception of baseline left ventricular developed pressure, which was modestly lower in the Fos-Post and PC groups than in control rabbits (P<0.05), there were no differences in hemodynamic parameters among the 4 groups of hearts (Table 1). Both ascorbate and fostriecin (plus ascorbate) reduced coronary flow similarly. The former elicited a small but significant decrease in heart rate (P<0.05). There were no differences in body weight, heart weight, or risk zone sizes among the groups (Table 2). Infarct sizes normalized as a percentage of the ischemic (risk) zone are shown in Figure 1. Hearts pretreated with fostriecin had only 8.4±1.8% infarction compared with 32.9±2.0% in control hearts (P<0.001). This level of protection is comparable to that seen with ischemic preconditioning (9.3±1.8%; P<0.001 versus control) and was not related to the presence of 3.4 μmol/L ascorbate, which had no effect on infarct size (30.0±2.5%; P=NS versus control). When fostriecin was given 10 minutes after the onset of occlusion, protection was still present, with 18.4±2.7% infarction (P<0.05 versus control). Although this was less protection than seen with pretreatment, infarcts were still 44% smaller than those in the untreated hearts.

TABLE 2. Infarct Size Data

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight, kg</th>
<th>Heart Weight, g</th>
<th>Risk Zone, cm²</th>
<th>Infarction, cm³</th>
<th>Infarction, % of Risk Zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>2.0±0.1</td>
<td>6.0±0.3</td>
<td>1.12±0.07</td>
<td>0.37±0.03</td>
<td>32.9±2.0</td>
</tr>
<tr>
<td>PC</td>
<td>6</td>
<td>2.1±0.0</td>
<td>6.7±0.3</td>
<td>1.02±0.07</td>
<td>0.10±0.01</td>
<td>10.0±1.5*</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>6</td>
<td>2.1±0.1</td>
<td>5.9±0.2</td>
<td>0.87±0.08</td>
<td>0.26±0.03</td>
<td>30.0±2.5</td>
</tr>
<tr>
<td>Fos-Pre</td>
<td>6</td>
<td>2.2±0.1</td>
<td>6.1±0.3</td>
<td>0.92±0.11</td>
<td>0.08±0.02</td>
<td>8.4±1.8*</td>
</tr>
<tr>
<td>Fos-Post</td>
<td>6</td>
<td>2.1±0.0</td>
<td>5.7±0.3</td>
<td>1.04±0.10</td>
<td>0.19±0.03</td>
<td>18.4±2.7†</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1. Values are mean±SEM. *P<0.001, †P<0.05 vs control.
Improved compared with the control cells (52.3±1.7% · h versus 64.4±1.6% · h, respectively; P<0.01). In the presence of either fostriecin or okadaic acid, cells survived significantly better as well (52.4±2.2 and 47.7±1.7% · h, respectively; P<0.01 versus control). Treatment of the cells with ascorbate alone did not improve survival of the cardiomyocytes (data not shown). Addition of 10 μmol/L fostriecin to oxygenated cells over a 3-hour time period had no effect on their survival (data not shown).

**PP Measurements**

Fostriecin inhibits the divalent cation–independent PP activity (PP1 and PP2A) contained in dilute homogenates of heart in a dose-dependent manner (Figure 3). The curve suggests that at least 2 fostriecin-sensitive PPs are present in the homogenate. The initial decline and then plateau of PP activity observed between 10⁻⁸ and 10⁻⁶ mol/L fostriecin represents successful inhibition of a more fostriecin-sensitive PP, whereas inhibition of the remaining PP activity requires considerably higher fostriecin concentrations, suggesting the existence of at least 1 additional, less sensitive PP. This pattern is similar to that reported previously in RINm5F cells and is consistent with studies conducted with purified PP1 and PP2A.⁹ Incubation of isolated cardiomyocytes (n=2) with 1 or 10 μmol/L fostriecin for 15 minutes inhibited PP2A activity by 67% and 94%, respectively, without any effect on PP1 activity (−2% and 7% inhibition for 1 and 10 μmol/L, respectively). Accordingly, for infarct size experiments, a concentration of 1 μmol/L fostriecin was used. This correlates with the amount needed to completely inhibit the activity of purified PP2A with little, if any, effect on PP1.

The baseline hemodynamic data after the equilibration period showed no differences in basal heart rate, developed pressure, or coronary flow between the 2 animal groups from which biopsies were obtained. After the onset of ischemia, there appeared to be a small time-related decline in PP2A activity in both control and preconditioned hearts (Figure 4A), although the change was significant only in the control group (P<0.01). More importantly, there was no difference in the percentage of stained cells vs control myocytes during 3 hours of simulated ischemia.

**Myocyte Studies**

Figure 2 shows the percentage of stained cells that were unable to exclude trypan blue dye in a hypotonic medium and were considered to be dead. Fewer preconditioned cells were stained after 180 minutes of simulated ischemia compared with control cells, thus indicating greater survival. Treatment with either of the 2 PP inhibitors, fostriecin or okadaic acid (data not shown), markedly protected the cells to an extent similar to that observed in preconditioned cells. For statistical comparison, the areas under the curves were calculated. Survival of the preconditioned myocytes was significantly improved compared with the control cells (52.3±1.7% · h versus 64.4±1.6% · h, respectively; P<0.01). In the presence of either fostriecin or okadaic acid, cells survived significantly better as well (52.4±2.2 and 47.7±1.7% · h, respectively; P<0.01 versus control). Treatment of the cells with ascorbate alone did not improve survival of the cardiomyocytes (data not shown). Addition of 10 μmol/L fostriecin to oxygenated cells over a 3-hour time period had no effect on their survival (data not shown).

**Phosphatase Inhibitor and Ischemic Preconditioning**

Figure 1. Infarct size normalized as % of ischemic (risk) zone in isolated perfused rabbit hearts. Control hearts (n=6) received only 30 minutes of regional ischemia. Hearts were ischemically preconditioned by 5 minutes of global ischemia followed by 10 minutes of reperfusion before 30 minutes of regional ischemia (PC; n=6). Either hearts were pretreated with 1 μmol/L fostriecin for 15 minutes before and during regional ischemia (Fos-Pre; n=6) or 10 μmol/L fostriecin was added to buffer 10 minutes after onset of ischemia for remaining 20 minutes of regional ischemia (Fos-Post; n=6). Ascorbate 3.4 μmol/L (present in fostriecin stock solution) was infused 15 minutes before and during regional ischemia and served as vehicle control (n=6). Pretreatment with fostriecin as well as ischemic preconditioning resulted in a significant reduction of infarction in risk zone vs control hearts (*P<0.01). Treatment after onset of regional ischemia still significantly reduced infarct size (**P<0.001). Ascorbate alone had no protective effect on infarct size.

**Figure 2.** Percentage of stained cardiomyocytes unable to exclude trypan blue in a hypotonic medium, an index of osmotic fragility. Shown are results of 4 separate myocyte isolations. Control cells (○) were pelleted for 3 hours to simulate ischemia. Metabolic preconditioning (□) was performed by incubating cells in glucose-free medium before simulated ischemia. PP inhibition was performed by adding 10 μmol/L fostriecin (△) to the cells 15 minutes before simulated ischemia. Oxygenated control (△) and metabolically preconditioned (□) cells without pelleting were incubated in oxygenated buffer over 210 minutes. Metabolic preconditioning and PP inhibition with fostriecin significantly reduced percentage of stained cells vs control myocytes during 3 hours of simulated ischemia.

**Figure 3.** Divalent cation–independent PP activity (expressed as % of PP activity without fostriecin) of dilute myocardial homogenate as a function of increasing concentrations of fostriecin measured in 6 independent experiments. Plateau between 10⁻⁸ and 10⁻⁶ mol/L fostriecin may be due to inhibition of PP2A. At 10⁻⁴ to 10⁻³ mol/L, fostriecin completely inhibited remaining divalent cation–independent PP activity (including PP1).
between the 2 groups. In contrast, PP1 activity (Figure 4B) declined in both the control and preconditioned hearts during the 60-minute period of ischemia ($P < 0.005$). But again, there was no apparent difference in activity in preconditioned and control hearts at the time points tested. Because sequential measurements with 2 independent groups of subjects provide a very weak statistical design,$^{15}$ we calculated the area under the time × phosphatase activity curve for each heart and compared the areas between the 2 groups (Figure 5, left). Again, there were no group differences.

The PP activity was also not different in control and preconditioned cardiomyocytes (Figure 6). In control cells, PP2A activity (Figure 6A) declined during 90 minutes of simulated ischemia ($P < 0.05$), although there was little change for the first 60 minutes and a modest fall during the final 30 minutes. In preconditioned cells, there was no effect of the duration of ischemia on enzyme activity ($P = 0.18$). In contrast, PP1 activity (Figure 6B) declined steadily after the onset of simulated ischemia in both groups ($P < 0.005$). PP1 and PP2A activities in oxygenated control and preconditioned myocytes without pelleting (data not shown) were unchanged during 90 minutes of incubation in oxygenated buffer. Figure 5 (right) shows the area under the time × phosphatase activity curves for cardiomyocytes. Again, there was no difference in the activities of PP1 and PP2A in control and preconditioned myocytes.

### Discussion

The present study demonstrates that fostriecin, a potent and selective inhibitor of PP2A,$^9$ was highly protective against infarction in the rabbit heart. Although the protection was equivalent to that of ischemic preconditioning, there was no indication that either PP1 or PP2A was inhibited in the ischemically preconditioned heart, suggesting that protection by preconditioning is not directly mediated by either of these 2 PPs. It is important to note that fostriecin was still protective even when given after the onset of ischemia, which is different from what we have found with other preconditioning mimetics such as adenosine.$^{16}$ Infarction progresses $\approx 5$-fold faster in rabbit than primate heart.$^{17}$ Thus, administration of fostriecin as late as 50 minutes after the onset of symptoms in humans might be expected to offer protection equivalent to that seen in the rabbits receiving the drug 10 minutes after coronary occlusion.

PP1 and PP2A, members of a large family of serine/threonine phosphatases, are well characterized, and they are both present in quantity in virtually all tissues.$^{14}$ Still, relatively little is known about PPs in the heart. Ingebritsen et al.$^{18}$ described PP1 and PP2A activity in homogenates of rabbit hearts and noted that the amount of PP2A was $\approx 3$ times
larger than that of PP1, comparable to the differences in specific PP activity that we observed in our biopsies. Quintaje et al.\(^9\) showed that PP2A activity is present in rat cardiomyocytes as well. The baseline PP1 activity was higher in myocytes (Figure 6) than in left ventricular biopsies from intact hearts (Figure 4). This difference may be due to the contamination of biopsies with nonmyocardial cells.

The assay system may be affected by other PPs in the homogenate. Although PP2B and PP2C should have been inhibited, we cannot exclude the possibility that the recently identified okadaic acid–sensitive PP4, PP5, and PP6\(^{14,20}\) might also be present and could have contributed to the PP2A and PP1-like activity.

Armstrong et al.\(^{6,6}\) used both okadaic acid and calyculin A to protect cardiomyocytes, but at concentrations that would have inhibited many of the PPs of the cell. These inhibitors not only delayed the appearance of osmotic fragility of the oxygen-deprived cells but also increased their rate of contracture, indicating an accelerated rate of ATP depletion. The ATP depletion probably resulted from the nonspecific inhibition of PPs other than PP2A, because fostriecin did not accelerate contracture in our cells. It is assumed that inhibition of PP2A accounted for protection by fostriecin, because the concentration of drug used was too low to affect PP1. However, because the effects of fostriecin on PP4, PP5, and PP6 are currently unknown, the contribution of inhibition of these other PPs cannot be discounted.

When the protective effect of PP inhibitors was first described, it was attributed to preservation of constitutively phosphorylated phosphate groups on cytoskeletal proteins.\(^8\) It was conjectured that dephosphorylation during deep ischemia would result in a loss of cytoskeletal integrity, causing the cell to rupture when subjected to the osmotic stress associated with reperfusion. One interesting possibility might be that during ischemic preconditioning, a signal transduction pathway is activated that terminates in inhibition of PPs, which in turn would protect by preservation of phosphorylated cytoskeletal proteins. The PP measurements, however, did not support the hypothesis that protection by ischemic preconditioning is the result of inhibition of either PP2A or PP1 activity in the rabbit heart. Unless the effect of preconditioning is to inhibit one of the other PPs that we did not measure, the most likely unifying explanation is that PP inhibitors protect simply by promoting the phosphorylation of the substrate of some kinase in the protective signal transduction pathway. Indeed, fostriecin has been reported to inhibit dephosphorylation of PKC-specific substrate.\(^{21}\) Because we have found that PKC must phosphorylate substrate during the prolonged ischemic period to protect,\(^{22}\) augmentation of the effect of that phosphorylation by a phosphatase inhibitor started after the onset of ischemia would be expected to be protective.

Whereas 1 \(\mu\)mol/L fostriecin was effective when given as pretreatment, we used a 10-\(\mu\)mol/L concentration of fostriecin in the posttreatment experiments. Collateral flow is known to be very low in the rabbit heart.\(^{21}\) We reasoned that increasing the concentration of fostriecin 10-fold in the perfusate would decrease by a factor of 10 the time required to load the tissue via the collateral vessels. We did not test whether the increased concentration was essential.

Fostriecin has been used clinically and has few reported side effects in humans.\(^{24}\) It was initially tested as an antitumor agent on a daily schedule for 5 days in a phase I clinical trial.\(^{24}\) Subsequently, fostriecin was shown to be an inhibitor of PP2A and PP1.\(^{7}\) Okadaic acid, another PP2A inhibitor, is quite toxic.\(^{7,13,14}\) but the clinical experience with fostriecin would suggest that the toxicity of okadaic acid is unrelated to its activity against PP2A. Because of the relatively mild side effects of fostriecin and because it is protective even when started after the onset of ischemia, it might be an agent that could be administered very early to patients presenting with chest pain of unknown origin on the chance that the pain might have originated from ischemic myocardium. In those patients actually having an acute myocardial infarction, early treatment should result in a powerful anti-infarct effect.

Acknowledgments

This study was supported in part by grants HL-20648 and HL-50688 from the National Institutes of Health, NHLBI, and CA-60750 from the National Cancer Institute. Dr. Weinbrenner is sponsored by a grant from the Deutsche Forschungsgemeinschaft (We 1955/1-1).

References

Fostriecin, an Inhibitor of Protein Phosphatase 2A, Limits Myocardial Infarct Size Even When Administered After Onset of Ischemia

Christof Weinbrenner, Christopher P. Baines, Guang-Shung Liu, Stephen C. Armstrong, Charles E. Ganote, Aimée H. Walsh, Richard E. Honkanen, Michael V. Cohen and James M. Downey

*Circulation*. 1998;98:899-905
doi: 10.1161/01.CIR.98.9.899

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/98/9/899

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/